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ERβ-dependent effects on uterine endothelial cells are cell specific and mediated via Sp1

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STUDY QUESTION: What are the *in vitro* effects of estrogen receptor β (ERβ) activation on the function of endothelial cells (ECs) from different vascular beds: human endometrial ECs (HEECs; endometrium), uterine myometrial microvascular ECs (UtMVECs; myometrium) and human umbilical vein ECs (HUVECs)?

SUMMARY ANSWER: Studies conducted *in vitro* demonstrate that the ERβ agonist 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN) has EC type-specific effects on patterns of gene expression and network formation. Identification of a key role for the transcription factor Sp1 in ERβ-dependent signaling in uterine ECs offers new insights into cell-specific molecular mechanisms of estrogen action in the human uterus.

WHAT IS KNOWN ALREADY: Estrogens, acting via ERs (ERα and ERβ), have important, body-wide impacts on the vasculature. The human uterus is an estrogen target organ, the endometrial lining of which exhibits physiological, cyclical angiogenesis. In fixed tissue sections, human endometrial ECs are immunopositive for ERβ.

STUDY DESIGN, SIZE, DURATION: Cells were treated with a vehicle control or the ERβ agonist, DPN, for 2 h or 24 h ($n = 5$) followed by gene expression analysis. Functional assays were analyzed after a 16 h incubation with ligand ($n = 5$).

PARTICIPANT/MATERIALS, SETTING, METHODS: Analysis of DPN-treated ECs using Taqman gene array cards focused on genes involved in angiogenesis and inflammation identified cell type-specific ERβ-dependent changes in gene expression, with validation using qPCR and immunohistochemistry. Molecular mechanisms involved in ERβ signaling were investigated using bioinformatics, reporter assays, immunoprecipitation, siRNA and a specific inhibitor blocking Sp1-binding sites. The endometrium and myometrium from women with regular menses were used to validate the protein expression of candidate genes.

MAIN RESULTS AND THE ROLE OF CHANCE: HEECs and UtMVECs were ERβ+/ERα−. Treatment of ECs with DPN had opposite effects on network formation: a decrease in network formation in HEECs ($P \leq 0.001$) but an increase in UtMVECs ($P \leq 0.05$). Genomic analysis identified opposite changes in ERβ target gene expression with only three common transcripts (*HEY1*, *ICAM1*, *CASP1*) in all three ECs; a unique profile was observed for each. An important role for Sp1 was identified, consistent with the regulation of ERβ target genes via association with the transcription factor ('tethered' mechanism).

LIMITATIONS, REASONS FOR CAUTION: The study was mainly carried out *in vitro* using ECs of which one type was immortalized. Although the analysis of the protein expression of candidate genes was carried out using intact tissue samples from patients, investigations into *in vivo* angiogenesis were not carried out.

WIDER IMPLICATIONS OF THE FINDINGS: These results have implications for our understanding of the mechanisms responsible for ERβ-dependent changes in EC gene expression in hormone-dependent disorders.

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Key words: uterus / estrogen / endothelial / angiogenesis / Sp1

Introduction

Estrogens have body-wide effects and are essential regulators of reproductive function in part by modulating key processes such as angiogenesis and inflammation (Smith, 2001; Jabbour et al., 2009). Endothelial cells (ECs) that line the interior surface of blood vessels are believed to be direct targets for estrogen action. Notably, both positive and negative effects of estrogenic ligands (natural and synthetic) on vascular function have been reported. These include comparisons between the incidence of vascular disease in men and women (Farhat et al., 1996; Vitale et al., 2010), in women before and after the menopause (McCrohon et al., 2000) and in women taking hormone replacement therapy (Yang and Reckelhoff, 2011). Angiogenesis is tightly regulated during development and in adulthood. In adults, physiological angiogenesis is a feature of reproductive tissues subject to cyclical remodeling in response to sex steroids (Jabbour et al., 2006) and is an essential component of normal wound healing (Bao et al., 2009). In contrast, aberrant angiogenesis is associated with disorders of the reproductive system (Smith, 2001) and with tumour growth and metastasis (Weis and Cheresh, 2011).

The human uterus contains two distinct tissue layers, the outer muscular myometrium and the inner multi-cellular endometrium. In a normal non-pregnant woman, both layers are exposed to cyclical variations in circulating concentrations of estrogens arising from ovarian activity (Abraham, 1974). The inner/luminal layer of the endometrium is shed at the time of menses and regeneration, followed by growth of new blood vessels, which is an essential feature of the estrogen-dominated proliferative phase (Nayak and Brenner, 2002). There have been conflicting reports of the impact of estrogenic ligands on endometrial angiogenesis (Girling and Rogers, 2005). The limited number of studies that have been carried out on purified cell populations suggest that estrogens may stimulate angiogenic activity of both endometrial (Kayisli et al., 2004) and myometrial ECs (Zaitseva et al., 2004), although the study on myometrial ECs included cells isolated from women with fibroids, a patient group in which endometrial function may be disturbed (Sunkara et al., 2010).

In the uterus as in other tissues, estrogen action is mediated by receptors acting as ligand-activated nuclear transcription factors or as part of membrane-associated signaling cascades (Heldring et al., 2007). Estrogen-dependent changes in gene expression can be mediated by binding of estrogen receptors (ERs) to DNA either directly via classical mechanisms at estrogen response elements (EREs), or through tethered (non-classical) associations with other transcription factors (e.g. Sp1, Jun/Fos), involving half EREs and GC-rich or AP-1 regions, respectively (O'Lone et al., 2004). Human ERs are the products of two genes *ESR1* and *ESR2* that encode the ERα and ERβ proteins, respectively. These ER subtypes exhibit differential temporal and spatial expression patterns within reproductive tissues and these profiles have impacts on tissue function (Gibson and Saunders, 2012). ERα and ERβ have a similar arrangement of domains including a highly conserved DNA-binding domain and a ligand-binding domain (LBD; Matthews and Gustafsson, 2003). The LBD of both receptors has been crystallized and differences in the size/shape of the ligand-binding pocket have led to the development of synthetic subtype-selective ER agonists, examples include 4,4′,4′-(4-propyl-(1H)-pyrazole-1,3,5-tryl)trisphenol (PPT, ERα selective) and 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN, ERβ selective; Sun et al., 2003). Ligand binding induces a conformational change in the receptor, unique to the ligand–receptor combination. The resultant 3D structure determines which co-regulatory proteins are bound to the complex; this can play an important role in determining whether target gene expression is augmented or abrogated (Nilsson and Gustafsson, 2010). Additionally, a number of anti-estrogens that block receptor activation have been developed, e.g. the anti-estrogen ICI 182780 (Fulvestrant), which functions as an estrogen receptor down-regulator, it binds ERα and ERβ with high affinity, blocks receptor dimerization and accelerates receptor degradation (Hermenegildo and Cano, 2000).

Studies on the relative contributions of ERα and ERβ to body-wide impacts of estrogens have revealed that ERα plays a key role in the regulation of cell proliferation and stromal–epithelial interactions and that co-expression of ERβ in ERα-positive cells can alter the pattern of gene expression (Gustafsson, 2003; Hewitt et al., 2005).

Studies have shown that ECs behave in a vascular bed specific manner in response to locally derived signals (Rocha and Adams, 2009). This implies that studies conducted on EC function must be carried out on cells derived from the vascular bed of interest. As estrogen also exhibits tissue selective effects, the commonly used human umbilical vein EC (HUVEC) model could potentially be inappropriate to model uterine EC function. To date, there is little evidence as to the function of ERβ when it is the sole ER subtype present in cells. We have previously reported that endometrial ECs are ERβ+/ERα− (Critchley et al., 2001) suggesting estrogen-dependent effects on their function are ERβ mediated.

The aim of the current study was to investigate the functional consequence of ligand-dependent ERβ activation in ECs from different compartments of the human uterus.

Materials and Methods

Cells and tissues

Three EC lines were used in the current study. The human endometrial ECs (HEECs; gifted from Yale University) were originally isolated from human endometrial microvessels using Ulex europaeus lectin (Schatz et al., 2000). These cells were subsequently telomerase immortalized and a comparison between the primary and immortalized cells demonstrated that they retained an identical phenotype including the expression of CD31, von Willebrand's factor and the Tie-2 receptors (Schatz et al., 2000; Krikun et al., 2005a). Primary uterine myometrial microvascular ECs (UtMVECs) were obtained from Lonza (Walkersville, USA); these cells are guaranteed through 15 population doublings and sold as CD31/105 and von Willebrand Factor VIII positive. HUVECs (gifted from T. Ramaesh, University of Edinburgh) are commercially available primary ECs that are widely used for cell-based research into factors regulating EC function. HUVECs were included in the current study as a control (non-uterine) EC. All ECs were maintained in endothelial growth media (EGM-2) (Lonza) supplemented with 10% fetal calf serum (FCS) in flasks coated with attachment factor (Gibco, Paisley, UK). Ishikawa cells (endometrial adenocarcinoma cell line; European collection of cell cultures (UK) were maintained in Dulbecco's modified eagle's medium (Gibco) supplemented with 10% FCS, 2 mmol/l, L-Glutamine, antibiotics and non-essential amino acids. Cells were cultured at 37°C with 5% CO_2; at least 24 h prior to experiments, medium was changed to phenol red-free media with 10% charcoal stripped FCS. Cells were stimulated with 10^{-8} M 17β-estradiol (E2; Sigma, UK), the ERα-selective agonist PPT

(Tocris, Bristol, UK) or the ERβ-selective agonist DPN (Tocris) alone or in combination with the anti-estrogen Fulvestrant—ICI 182 780 (ICI; 10^{-7} M; Tocris) dissolved in dimethylsulphoxide (DMSO). Full-thickness uterine biopsy material used for immunohistochemistry (IHC) was obtained as previously described (Critchley *et al.*, 2001).

Immunodetection

Immunocytochemistry was carried out on cells grown on chamber slides. Cells were fixed in ice-cold methanol for 10 min and then permeabilized in a blocking solution containing 0.2% IGEPAL (Sigma-Aldrich). Non-specific binding sites were blocked with a species-specific blocking solution [1:5 part normal serum in Tris-buffered saline (TBS)/5% bovine serum albumin (BSA)] for 30 min. Endogenous streptavidin and biotin were blocked using a kit available from Vector Laboratories. Primary antibodies were diluted in blocking solution (Supplementary data, Table S3) and incubated overnight at 4°C. Biotinylated secondary antibodies (1:500) were diluted in 5% BSA in TBS and incubated at room temperature (RT). A streptavidin–HRP conjugate (1:1000; Sigma-Aldrich) was diluted in TBS and used for incubation at RT for 30 min followed by visualization with ImmPACT™ DAB peroxidase substrate (Vector Laboratories). Cells were counterstained, dehydrated, cleared in xylene and mounted in Pertex.

IHC was carried out on paraffin-embedded full-thickness uterine sections. Sections were dewaxed in xylene and rehydrated to water. Citrate antigen retrieval was performed followed by endogenous peroxidase block with 3% H_2O_2 in methanol. Streptavidin–biotin block (Vector Laboratories) was carried out followed by species-specific block and incubation with primary antibody overnight. Secondary antibody detection and counterstaining were performed as above. For full-thickness sections, tiling was carried out using Axiovision for Axiovert (Carl Zeiss). Dual immunofluorescence on paraffin-embedded full-thickness uterine sections was achieved by dewaxing and rehydrating sections as before, then the antigen retrieval and peroxidase block were performed followed by species-specific block. All washes were carried out in PBS for florescent methods. Primary antibody was diluted in blocking solution and incubated overnight at 4°C. A secondary F (ab) polyclonal antibody to IgG (HRP) was diluted in blocking solution and incubated on sections for 30 min at RT. All subsequent washes included a single wash with PBS containing 0.05% Tween and then a wash in PBS. Antibody detection was carried out using a TSA™ system kit labelled with either Cy3 (red) or fluorescein (green; Perkin Elmer, Inc.) diluted 1:50 for 10 min. For the detection of the second protein of interest, sections were microwaved for 2.5 min in boiling citrate buffer, an additional species-specific block was carried out and the second primary antibody was applied overnight at 4°C. The secondary antibody was detected as before with the appropriate TSA system and sections were counterstained with DAPI (1:500) for 10 min. Slides were mounted in Permafluor (Thermo Fisher Scientific) and imaged using an LSM710 confocal microscope and AxioCam camera (Carl Zeiss, Inc.).

ER proteins were detected using a mouse monoclonal raised against a peptide present at the C-terminus of full-length wild-type ERβ (ERβ1; Serotec, Kidlington, UK) but absent from splice variants of human ERβ (ERβ2, ERβ5; Critchley *et al.*, 2002) and a mouse monoclonal specific for ERα (Vector, Peterborough, UK). CD31 protein was detected using a mouse monoclonal anti-CD31 (Dako, Cambridgeshire, UK). Candidate proteins were detected using a rabbit polyclonal anti-IFNB1 (Epitomics, CA, USA) and a rabbit monoclonal ICAM1 (Abcam, Cambridge, UK). More details on antibody dilutions are available in Supplementary data, Table S3.

RNA extraction and cDNA synthesis

Total RNA was extracted at 2 or 24 h post-stimulation with ligand using the RNAeasy kit (QIAGEN, Crawley, UK) and cDNA was synthesized using SuperScript®VILO™ (Invitrogen, Paisley, UK) with a starting template concentration of ~100 ng RNA.

Quantitative real-time PCR

Taqman array cards (TACs) for angiogenesis and inflammation gene signatures (Applied Biosystems, CA, USA) were analyzed according to the manufacturer's instructions using Taqman® Universal PCR mastermix (Applied Biosystems). Additional real-time PCR reactions were performed using the Roche Universal ProbeLibrary (Roche Applied Science, West Sussex, UK) and Express qPCR Supermix (Invitrogen). qPCR was performed on a 7900 Fast Real-Time PCR machine with 18S as the endogenous control. Primer sequences can be provided on request. Bioinformatics analysis was carried out using Metacore™ (GeneGo.Com).

Western blot analysis

Nuclear protein was prepared using a Nuclear Extract Kit (Active Motif, CA, USA) separated on a NuPAGE® Bis-Tris Gel (Invitrogen), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) and probed with the following antibodies: mouse polyclonal anti-ERβ (Santa Cruz Biotechnology, CA, USA), mouse monoclonal anti-ERα (Vector), mouse monoclonal anti-Sp1 (Abcam), rabbit polyclonal anti-Lamin β1 (Abcam) or mouse monoclonal anti-Lamin β1 (Abcam). For secondary detection, goat anti-mouse IgG (Alexa fluor IR 680, Invitrogen Molecular Probes) and goat anti-rabbit IgG (IR Dye 800 CW, LI-COR, NE, USA) were used at 1:10 000 dilution. Antibody binding was visualized using infra-red imaging on an Odyssey imaging system (LI-COR).

Network formation assay

ECs were plated at 25 000 cells per insert into transwells coated with phenol red-free growth factor reduced Matrigel (BD Biosciences, Oxford, UK). Where appropriate, the pure anti-estrogen Fulvestrant/Faslodex® (ICI) was added into the upper chamber of media 1 h before the addition of ligands. All ligands were added to the bottom chambers and cells were incubated for 16 h at 37°C with 5% CO_2. To block binding of Sp1 to GC regions, we used Mithramycin A (50 nM; Sigma). Cells were fixed in ice-cold methanol for 20 min and briefly stained with haematoxylin. The formation of networks was visualized using an Axiovert microscope (Carl Zeiss, Germany), where three independent fields of vision were captured at ×5 magnification for each well. Network formation was quantified using ImageJ (NIH.gov) software, with images converted to binary and the area of networks analyzed using the 'count particles' option. Results were verified by counting the number of closed polygons. The mean number of polygons per well was calculated, followed by the mean for each treatment. The fold change compared with the vehicle control was plotted.

Immunoprecipitation

Total cell protein was extracted from HEECs treated with DMSO or DPN (10^{-8} M) for 24 h. Immunoprecipitations were performed using Dynabeads protein G (Life Technologies) following the manufacturer's instructions with anti-ERβ (Abcam) or rabbit IgG (Dako) as the control. Antibodies were cross linked to the Dynabeads using BS3 (Thermo Fisher Scientific) and samples incubated with the cross-linked complex overnight at 4°C. The input and immunopreciptated proteins were resolved by SDS–PAGE electrophoresis and transferred onto PVDF membranes. Complexes containing Sp1 were detected by incubating membranes with mouse anti-Sp1 (Abcam) at 1:300 dilution and analyzed using the LICOR system as described above.

siRNA knockdown

ECs were transfected with a non-specific siRNA (negative) or a synthetic siRNA directed to *ERβ* or *Sp1* (Ambion, Paisley, UK) at a final concentration

of 5 nM using HiPerFect transfection reagent (QIAGEN). At 48 h after transfection, cells were treated with ligand and harvested at 2 or 24 h post-treatment. Depletion was confirmed by qPCR.

Proliferation assay

ECs were plated into 96-well plates at 3000 cells/well and allowed to adhere overnight. Cell medium was replaced with EGM-2 1% charcoal stripped FCS for 3 h, followed by addition of ligands. Treatments were replaced three times during the 72 h culture period. To assess proliferation, medium was removed and replaced with a 1:5 ratio of CellTitre96Aqueous One Solution Proliferation Reagent (Promega) and EGM-2 1%. After a 3 h incubation, the formation of formazan was recorded by measuring the absorbance at 490 nm.

Luciferase reporter assays

Cells were plated at 1×10^5 per well into 24-well plates and left to adhere overnight; for each luciferase experiment, a corresponding control plate was set up allowing the analysis of protein levels for normalization. Cells were infected with an adenoviral 3× ERE luciferase construct (in house development) with a multiplicity of infection of 50, with 6 μg/ml Polybrene (Sigma). For the adenoviral system, 24 h after incubation, cells were stimulated with ligands (10^{-8} M). Whole cell lysates were harvested 24 h after the addition of ligand with Glo Lysis buffer (Promega). Lysates were transferred to luminometer plates and a 1:1 ratio of Bright-Glo reagent (Promega) was added. Luminescence was measured using a Fluostar OPTIMA plate-reader (BMG Labtech). Analysis of corresponding plates was analyzed for protein concentration using the DC protein assay (Bio-Rad), and reporter gene expression was corrected by protein levels.

Statistical analysis

Statistical analysis was carried out using a one-way analysis of variance followed by a Neuman–Keuls post-comparison test or a two-tailed unpaired Student *t*-test. For qPCR data, statistical analysis was carried out on transformed data. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Ethical approval

In brief, a full-thickness uterine biopsy material was collected from women undergoing hysterectomy. Written informed consent was provided by all subjects and ethical approval for tissue collection was granted by the Lothian research ethics committee. Patients had regular menstrual cycles and were not taking exogenous hormones.

Results

Uterine ECs express ERβ but not ERα

To extend and confirm our previous observations, we used dual fluorescent IHC to co-stain full-thickness uterine samples for ERβ and the EC marker CD31. ERβ and CD31 were co-localized in ECs within the endometrium and myometrium at all stages of the menstrual cycle (Fig. 1A and B). Myometrial ECs appeared immunonegative for ERα even when closely adjacent cells were CD31+ (Fig. 1C and D; Supplementary data, Fig. S1A). Eight patient samples were analyzed in detail (four proliferative stage and four secretory stage), all vessels were examined in each section of the myometrium and no CD31-positive cells were found to express ERα. ERβ+ ECs were readily detectable in all uterine layers (Supplementary data, Fig. S1B). Cells derived from microvascular beds of the human endometrium (HEECs; Schatz *et al.*, 2000; Krikun *et al.*, 2005a,b), myometrium (UtMVECs) and HUVECs were characterized to confirm their EC phenotype (Supplementary data, Fig. S2). All EC models were ERβ+/ERα− at the level of mRNA (Fig. 1E) and protein (Fig. 1F). Ishikawa cells were used as an ERα expressing control to validate our antibodies and primers. These comprehensive profiling studies confirmed that uterine ECs recapitulated the pattern of expression of ERs in their native vascular beds.

A selective ERβ agonist has an endothelial subtype-specific impact on angiogenesis

An *in vitro* model of angiogenesis (the network formation assay) was used to compare the impacts of E2 and the ERβ-selective agonist DPN on endothelial function. These studies revealed striking opposite impacts of DPN on HEECs and UtMVECs with a significant decrease in the amount of networks formed by HEECs (Fig. 2A; $n = 6$, $P \leq 0.001$) and a significant increase in UtMVECs (Fig. 2B; $n = 6$, $P \leq 0.05$). In both, the effect was abrogated by the addition of the pure anti-estrogen Fulvestrant (ICI) and surprisingly the same concentration of E2 had no significant impact. In HUVECs, we found that E2 and DPN had no significant impact on network formation compared with the vehicle control; however, there was a significant decrease in network formation when cells were treated with ligand plus ICI (Fig. 2C; $n = 6$, $P \leq 0.01$ and $P \leq 0.001$). In HEECs and HUVECs, treatment with DPN or E2 increased cell proliferation (Supplementary data, Fig. S3A and C); the changes observed in UtMVECs were not significant (Supplementary data, Fig. S3B).

The ERβ-selective agonist DPN induces specific patterns of gene expression in ECs derived from different vascular beds

Using targeted gene arrays, we compared changes in the expression of genes implicated in the regulation of angiogenesis and inflammation in response to treatment with DPN between uterine ECs and a widely used EC model (HUVECs). DPN was used in TAC studies because this subtype-selective ER agonist produced the most profound effect in the above functional studies. We used two time points (2 and 24 h) to encompass both an early and later response to ERβ activation in cells. Analysis of RNA recovered from cells incubated with or without ligand at both time points resulted in the identification of significant (>1.5-fold) changes in 22 of 92 genes associated with angiogenesis (Fig. 3A; $n = 3$) and in 14 of 92 genes associated with inflammation (Fig. 3B; $n = 3$) as analyszed using TAC. The complete lists of differentially regulated genes in each cell type are given in Supplementary data, Tables S1 and S2. Notably, there were very few ERβ-dependent genes common to more than one of the three cell lines and several of the shared ERβ-dependent genes identified were regulated in opposite directions in HEECs and UtMVECs (Supplementary data, Table S1, Fig. 4). Analysis of the angiogenesis gene set revealed that only the expression of *HEY1*, a transcriptional repressor part of the Notch signaling family was altered in response to DPN (24 h) in all three EC lines (Fig. 3A). Two other genes were regulated in HEECs and UtMVECs but not in HUVECs; these were interferon β-1 (*IFNB1*) and autotaxin/ectonucleotide pyrophosphatase/phosphodiesterase 2 (*ENPP2*). Analysis using the inflammation TAC array revealed two ERβ-dependent transcripts common to all three ECs: intercellular adhesion molecule 1 (*ICAM1*, CD54) and Caspase 1 (*CASP1*); these were significantly changed after 2 h in HEECs and 24 h in UtMVECs and HUVECs. We also observed

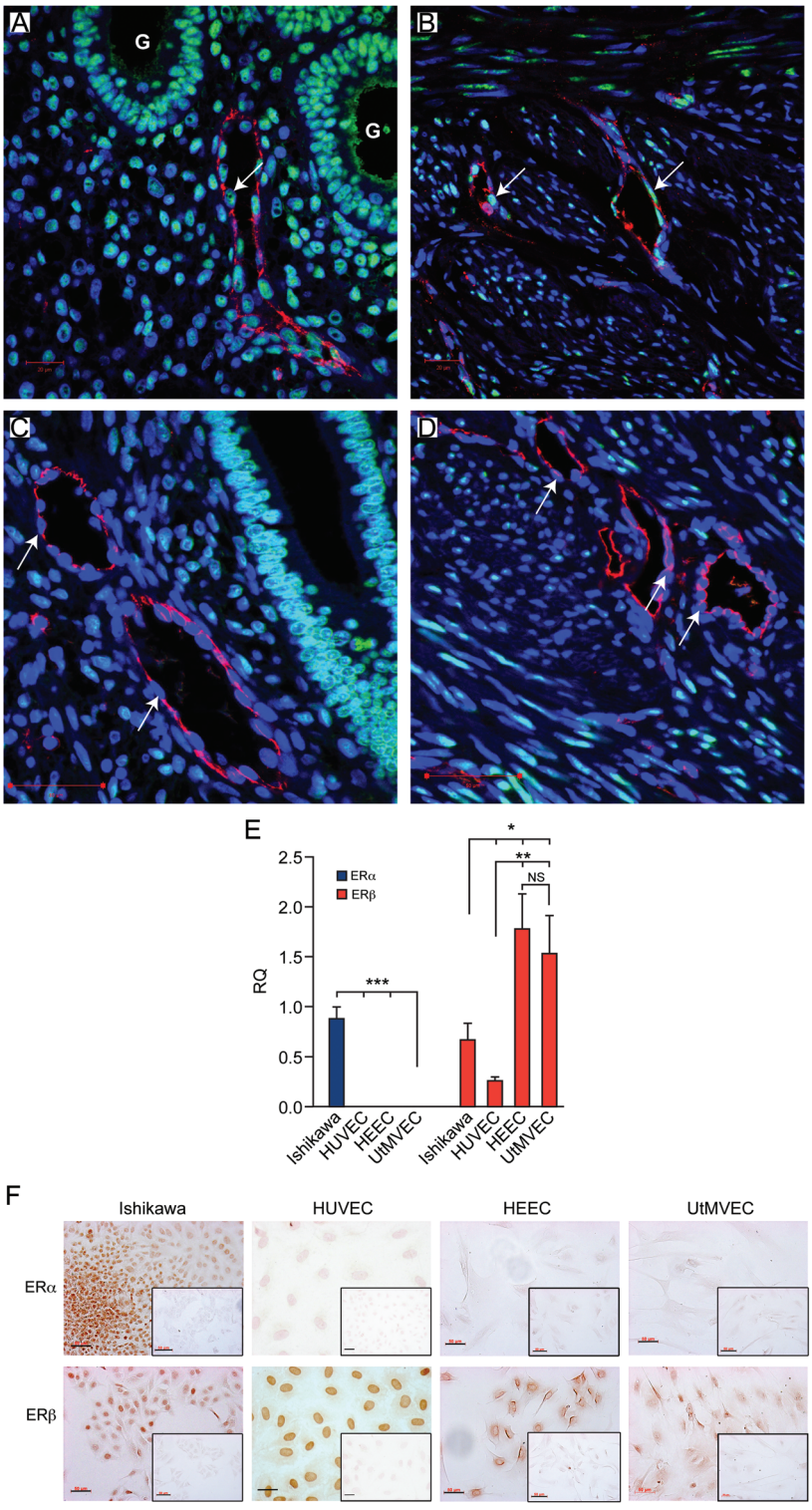

Figure 1 ERβ, but not ERα, is expressed in ECs in both the endometrium and myometrium and *in vitro* models retain the original phenotype. (**A** and **B**) Dual immunofluorescent staining for ERβ (green) and CD31 (EC marker; red) was carried out on full-thickness uterine biopsies at different stages of the menstrual cycle. Co-localization was observed in vessels in the endometrium (A) and myometrium (B). Arrows indicate ECs. G, Gland. Scale bar is 20 μm. (**C** and **D**) Dual immunofluorescent staining for ERα (green) and CD31 (red) carried out on full-thickness sections at different stages of the menstrual cycle. ECs were negative for ERα in the endometrium (C) and myometrium (D). Scale bar is 50 μm. (**E**) mRNAs encoding wild-type ERβ (ERβ1) and ERα were both detected in human adenocarcinoma cells (Ishikawa) but all three EC lines, HEECs, UtMVECs and HUVECs, only contained measurable concentrations of ERβ mRNAs. Values represent the mean ± SEM analyzed in triplicate from three separate experiments. Results are normalized to Ishikawa cells, where one replicate was given the arbitrary value of 1. RQ, relative quantification (*$P<0.05$, ***$P<0.001$). (**F**) Immunolocalization of ERα and ERβ1 carried out on cell lines grown on chamber slides—note only Ishikawa cells were ERα+. ERβ was localized to cell nuclei in all cell types. Negative controls with primary antibody omitted are shown as insets. Bar = 50 μm.

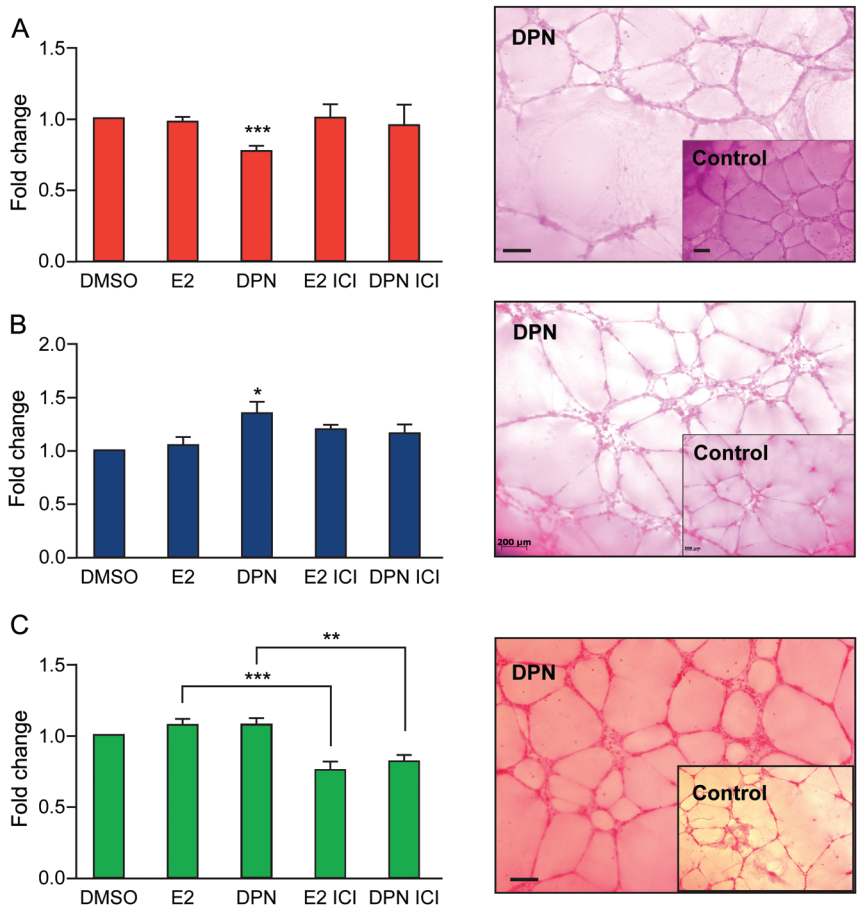

Figure 2 ERβ activation has specific and opposing effects on endometrial and myometrial EC function. (**A**, **B** and **C**) Cells were plated onto growth factor reduced matrigel and incubated with E2 or DPN (10^{-8} M) in the presence or absence of the pure ER antagonist (ICI; 10^{-7} M). The impact on network formation in HEECs (A), UtMVECs (B) and HUVECs (C) was analyzed after 16 h. Images show representative photos taken from wells treated with DPN (large images) or DMSO (inset) illustrating differences in the formation of networks. Scale bars are 200 µm. Values represent the mean ± SEM from five separate experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

particular trends present within the data set. For example, genes associated with prostaglandin synthesis were regulated in all three ECs. Although this common pathway was found, common genes within the pathway were not (Supplementary data, Table S2). To summarize, only one gene associated with angiogenesis and two genes associated with inflammation were regulated by DPN in all three EC lines. Interestingly, we found that the pattern of expression of angiogenic genes correlated with our functional studies. For example, in HEECs, we observed an overall down-regulation in pro-angiogenic factors but an up-regulation in angiogenesis inhibitors (Supplementary data, Table S1). This correlated with our observed decrease in network formation in response to DPN. However, in the UtMVECs, we observed an up-regulation in pro-angiogenic factors and a down-regulation in inhibitors in response to DPN, correlating with an increase in network formation.

To complement and extend the data gathered using the TAC arrays, qRT–PCR validation experiments were carried out using additional cultures of all three EC lines treated with DPN alone, DPN plus ICI, E2 alone, E2 plus ICI or the ERα-selective agonist PPT ($n = 5$; Fig. 4, Supplementary data S4 and S5). Changes in the expression of *HEY1* detected using TAC in response to DPN were confirmed using qRT–PCR in HEECs (significantly reduced) and UtMVECs (significantly increased). In all cases, inclusion of ICI abrogated the change observed and inclusion of PPT had no impact consistent with the lack of expression of ERα in these cells (Supplementary data S4A). Treatment of cells with E2 induced the same changes as DPN in UtMVECs and HUVECs but were opposite (increased expression) in HEECs (Supplementary data S4A). qRT–PCR analysis of *IFNB1* expression confirmed TAC results in HEECs (significantly up-regulated by DPN or E2 at 24 h). In UtMVECs, validation confirmed significant down-regulation of *IFNB1* by E2 but not by DPN (Supplementary data S4B). In HUVECs, *IFNB1* was significantly up-regulated following incubation with DPN or E2 for 24 h (Fig. 4B, Supplementary data S4B); this had not initially been detected using the TAC array. We were, therefore, able to recapitulate results gained using TAC (angiogenesis) in additional experiments, with the exception of *IFNB1* in UtMVECs that was regulated by E2 alone and not DPN.

Confirmation of expression of *ICAM1* in HEECs, UtMVECs and HUVECs in response to E2 or DPN as detected by qRT–PCR revealed

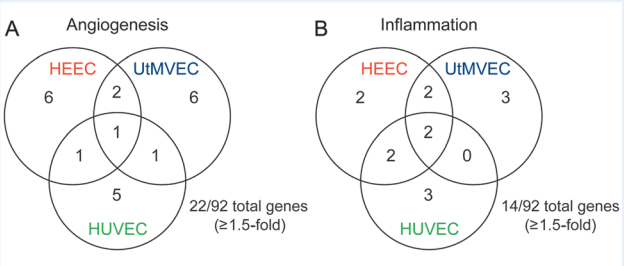

Figure 3 Incubation of ECs with DPN results in altered expression of distinct subsets of genes in ECs derived from different vascular beds. HEECs, UtMVECs and HUVECs were incubated with media containing vehicle alone (control, DMSO) or DPN (10^{-8} M in DMSO) for 2 or 24 h. A sample size of two from three separate experiments was generated for each time point and cell line; all samples were analyzed in parallel on TAC. Only genes that were subject to a statistically significant change following the treatment with DPN were reported. (**A**) Venn diagram representing ERβ-dependent genes associated with angiogenesis. (**B**) Venn diagram representing ERβ-dependent genes associated with inflammation. Diagrams represent the sum of all up- and down-regulated genes that showed statistically significant changes at 2 or 24 h post-stimulation. Full gene lists are supplied in Supplementary data, Tables S1 and S2. TAC, TaqMan array cards.

additional time dependent but similar impacts of DPN and E2, no significant response to PPT and abrogation of responses in the presence of ICI (Fig. 4C, Supplementary data Fig. S5A). qRT–PCR analysis of *CASP1* in HEECs (by E2) and HUVECs (by E2 and DPN) revealed an up-regulation at 2 h in contrast to the down-regulation detected by TAC (inflammation; Supplementary data Fig. S5A); this may indicate that ERβ-dependent regulation of *CASP1* is not as robust as other candidates. Validation of *CASP1* mirrored the TAC results for UtMVECs.

To complement studies using anti-estrogen, depletion of ERβ mRNA using siRNA was carried out in HEECs (Supplementary data Fig. S6A); incubation of ERβ-depleted cells with DPN failed to induce the same significant changes in *HEY1* and *IFNB1* mRNAs seen in cells incubated with a control siRNA (Supplementary data Fig. S6B and C).

To extend investigations on the expression of *IFNB1* and *ICAM1*, IHC was performed on full-thickness uterine sections. Both proteins were detected in CD31+ ECs within the endometrium (Supplementary data Fig. S7A and E; S7I and M) and myometrium (Supplementary data Fig. S7B and F; Fig. S7J and N), during both phases of the cycle. Both proteins were co-expressed with ERβ in ECs as well as in other uterine cell types including epithelial cells (Fig. S7 labelled G).

Bioinformatics identifies Sp1 as a key regulator of ERβ-mediated gene expression in ECs

Following the identification of ERβ-dependent changes in vascular function and associated changes in gene expression, additional studies were directed at understanding the molecular mechanisms by which ERβ induces changes in gene transcription in response to ligand activation. Endothelial and Ishikawa (control) cells were infected with an adenoviral construct containing a luciferase reporter gene under the control of a 3×ERE promoter. In Ishikawa cells, reporter gene expression was induced by E2, DPN and PPT and abrogated by the inclusion of ICI (Supplementary data Fig. S8A). In contrast, there was no evidence of reporter gene activation in any of the ERβ+ ECs (Fig. S8B–D). It has been proposed that ERβ-mediated transcription may involve 'tethered' mechanisms depending upon the recruitment of additional transcription factors; therefore, bioinformatic analysis of genes identified on the array cards was carried out. This revealed that 12 of 18 of the ERβ-dependent genes identified in HEECs, including *HEY1*, *ICAM1* and *ENPP2*, were associated with the transcription factor Sp1 (Fig. 5A). Western analysis confirmed the expression of Sp1 in all three ECs (Fig. 5B). Immunoprecipitation was performed on HEEC proteins using an anti-ERβ antibody with the detection of Sp1 in the complex confirmed following western blotting and probing the membrane with an anti-Sp1 antibody (Fig. 5C). This demonstrated that ERβ and Sp1 are bound together within HEECs regardless of the presence of ligand.

Inhibition of Sp1-dependent activity abrogates ERβ-dependent changes in EC function and gene expression

To investigate whether the ERβ-dependent changes in gene expression detected in EC were Sp1 dependent, we carried out siRNA knockdown experiments using HEECs. Partial knockdown of Sp1 mRNA was achieved using a Sp1-specific siRNA (Fig. 5D); the resulting reduction in the expression of Sp1 abrogated DPN-dependent changes in the expression of *HEY1* and *IFNB1* (Fig. 5E and F). To determine whether the ERβ-dependent decrease in network formation observed in HEECs was also Sp1 dependent, cells were treated with Mithramycin A, an anti-cancer drug that binds to GC-rich regions of chromatin and prevents binding of Sp transcription factors. Mithramycin A alone had no impact but the addition of Mithramycin A to cultures containing DPN rescued the cells from the ERβ-dependent decrease in network formation (Fig. 5G and H).

Discussion

This study is a comparative analysis of the impact of ERβ activation, via the binding of an ERβ agonist (DPN), on EC function and associated gene expression changes in microvascular EC lines derived from different vascular beds of the human uterus (HEECs and UtMVECs) and ECs obtained from HUVECs. HUVECs were included in the current study as they are used extensively in studies on EC function.

While the study does possess limitations because primary tissue was not used, we believe that our results demonstrate a number of novel findings. First, to our knowledge, this study is the first to directly compare ECs from the endometrium and myometrium and to indicate opposing properties for the two. This indicates that ER selective agonists may have distinct effects in different vascular beds, which may have implications for the therapeutic application of ER subtype-selective agonists in hormone responsive disorders characterized by aberrant angiogenesis. Secondly, our findings expand suggestions that ECs from different vascular beds exhibit unique phenotypes. Notably, we found little overlap in the expression of genes associated with angiogenesis and inflammation in the three ECs we analyzed, each of which demonstrated unique ERβ-dependent patterns of gene expression, suggesting that our cell models retain the phenotype of their vascular origin. These results also suggest that there may be no 'ideal EC model' in which to study ERβ-mediated changes in cell function and that assessment of the likely impacts of

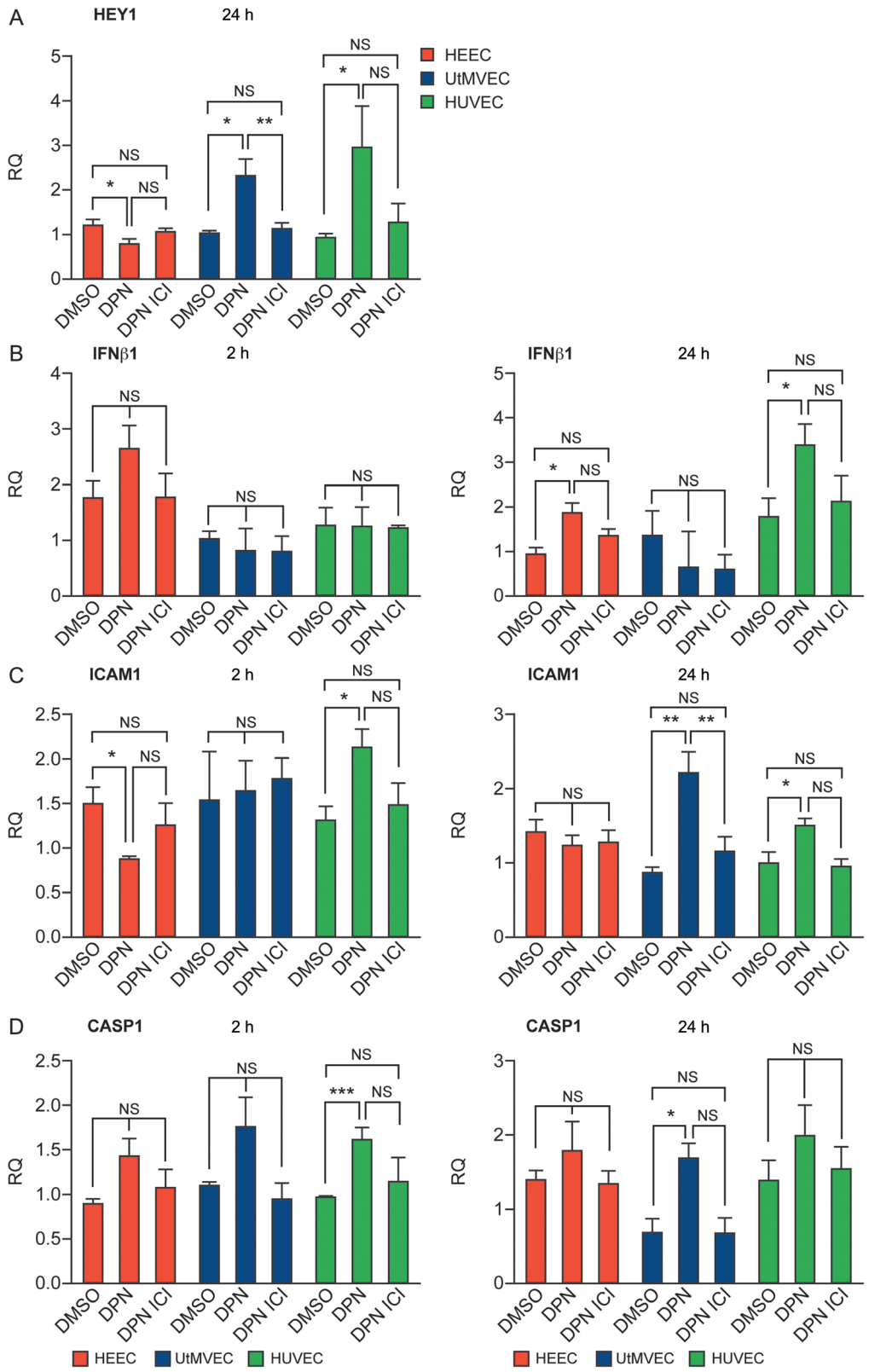

Figure 4 ERβ-dependent changes in gene expression are regulated in opposite directions in ECs derived from different vascular beds. qRT–PCR validation of TAC results was carried out using Roche Universal ProbeLibrary (Roche Applied Science) with Express qPCR Supermix (Invitrogen) on HEECs, UtMVECs and HUVECs, treated with the vehicle control (DMSO), DPN (10^{-8} M) or DPN plus the anti-estrogen Fulvestrant (ICI, 10^{-7} M). Values represent the mean ± SEM, $n = 5$. Results are normalized to the vehicle control where one replicate was given the arbitrary value of 1. RQ, relative quantification (*$P<0.05$, **$P<0.01$, ***$P<0.001$). (**A**) *HEY1* 24 h (no impact at 2h), (**B**) *IFNB1*, 2 and 24 h, (**C**) *ICAM1*, 2 and 24 h and (**D**) *CASP1*, 2 and 24 h. TAC, TaqMan array cards.

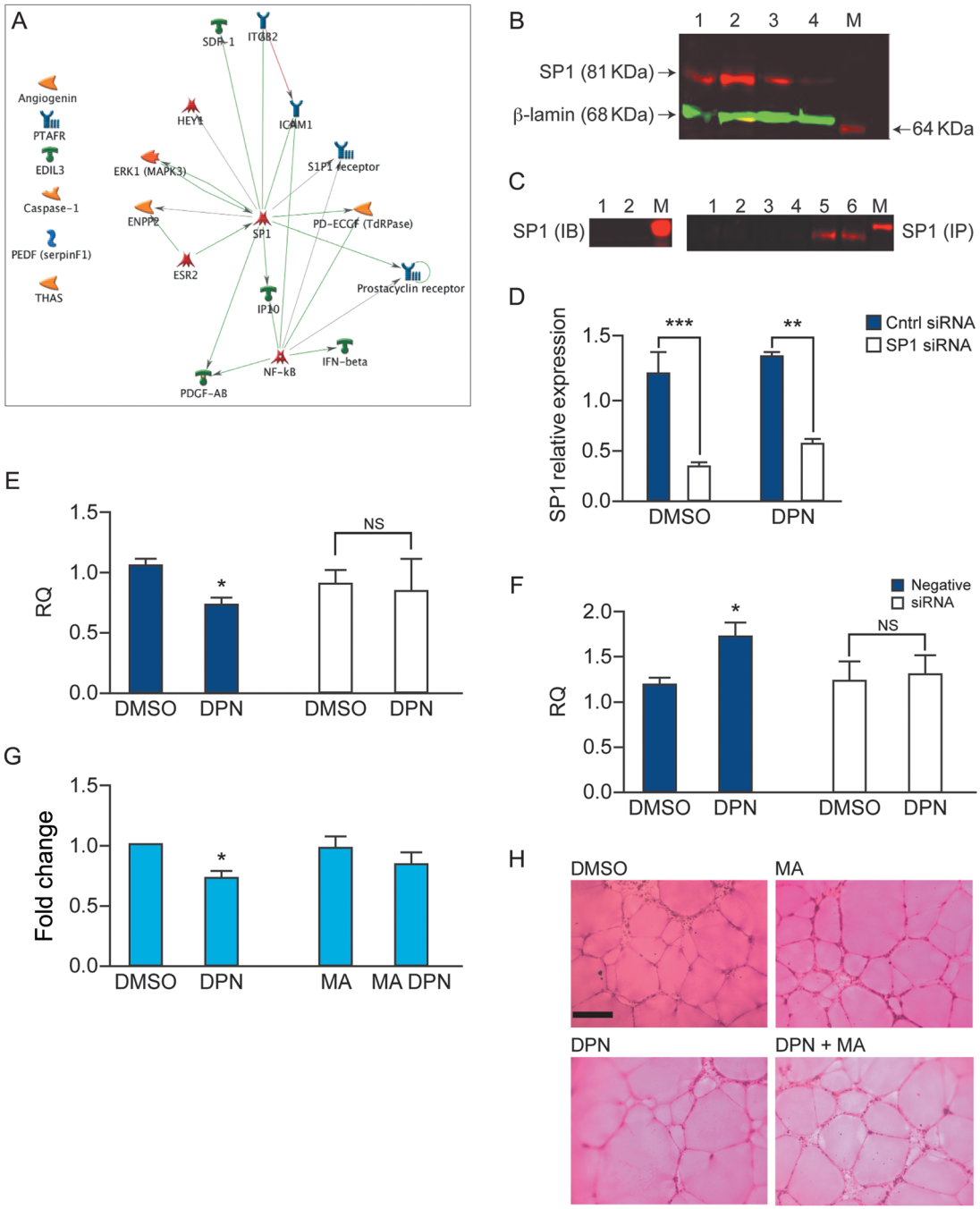

Figure 5 Sp1 is a regulator of ERβ-mediated gene expression and function in uterine ECs. (**A**) Bioinformatics analysis using Metacore™ revealed that 12 out of 18 ERβ-dependent genes identified in HEECs have known associations with the transcription factor Sp1. Metacore software utilizes some alternative gene names: S1P1 receptor (*ECGF*), PD-ECGF (platelet derived ECGF), prostacyclin receptor (*PTGIR*), IP10 (*CXCL10*), ESR2 (*ERβ*), ERK1 (*MAPK3*), THAS (*TBXAS1*), PEDF (*SERPINF1*), SDF1 (*CXCL12*). (**B**) Western blot analysis of nuclear extracts from all four cell lines using antibodies specific for Sp1 (81 KDa) and the endogenous control β lamin (68 KDa); lane 1, Ishikawa cells; lane 2, HUVEC; lane 3, HEEC; lane 4, UtMVEC; M, Marker. (**C**) Sp1 was detected by immunoblotting (IB) with Sp1-specific mouse monoclonal antibody. HEECs treated with DMSO (lane 1) or 10^{-8} M DPN (lane 2) for 24 h and after immunoprecipitation (IP) with an anti-ERβ rabbit polyclonal antibody in HEECs treated with DMSO (lane 5) or DPN (lane 6). No Sp1 was detected in HEECs immunoprecipitated with rabbit IgG (lane 3) or in the cross-linked IP sample (lane 4). (**D**) Knockdown of Sp1 in HEECs was achieved following transfection with a *Sp1*-specific siRNA (white bars) in the presence or absence of DPN for 24 h; control scrambled siRNA is shown with blue bars. qPCR analysis confirmed knockdown efficiency: 61% in DMSO-treated samples and 67% DPN-treated samples. (**E**, **F**) Knockdown of *Sp1* (white bars) in HEECs abrogated the significant impact of DPN (*$P<0.05$, **$P<0.01$, ***$P<0.001$). on the expression of *HEY1* (E) and *IFNβ1* (F), respectively (**G**) Network formation assay using HEECs showing that incubation with Mithramycin A (50 nM) rescued the DPN-mediated decrease in network formation. MA, Mithramycin A. $n = 6$. (**H**) Representative images for the different treatments reported in (G). Scale bar is 100 μm.

natural and synthetic estrogens on EC function needs to be conducted in models appropriate to the target tissue. Finally, this is the first study to suggest a role for Sp1 in ERβ-dependent changes in uterine EC function. Binding of liganded ERβ to Sp1 sites has been shown in cells expressing endogenous levels of ERβ (Vivar *et al*., 2010), and Sp1 has previously been implicated in uterine cell-specific expression of the HOXA10 gene in response to estrogen (Martin *et al*., 2007). However, this is the first study highlighting an important role for Sp1 in ERβ-directed transcription in ECs and drawing attention to the importance of ERE-independent, 'tethered' mechanisms in estrogen regulation of gene expression (Nilsson *et al*., 2001). Further studies are now merited to explore whether targeting Sp1-ERβ-dependent gene expression offers an alternative treatment option for targeting specific cell types in reproductive pathologies.

ERβ induces contrasting effects on uterine vascular function

There have been reports that myometrial ECs are heterogeneous in nature, that a subpopulation express ERα and ERβ and that ERα agonists promote angiogenesis in these cells (Zaitseva *et al*., 2004). We have not find this to be the case in present or past studies using full-thickness biopsies of normal human uteri; the myometrial EC model (UtMVECs) used in this study did not express ERα. Our robust characterization of ECs both *in situ* and in our chosen models failed to detect either ERα mRNA or protein in the EC lines. Of particular interest, was our finding that the ERβ-selective agonist, DPN, decreases network formation in endometrial ECs (HEECs). During the normal menstrual cycle, there is a significant increase in angiogenesis during the proliferative phase that replenishes the vascular bed. Although it may be presumed that E2 levels are responsible, the direct or indirect mechanism of the regulation of angiogenesis remains uncertain (Girling and Rogers, 2005). The endometrium is a complex multi-cellular tissue and we speculate that *in vivo*, an E2-dependent increase in angiogenesis may be mediated via ERα-positive cells such as perivascular cells, stromal fibroblasts or epithelial cells. For example, vascular endothelial growth factor (VEGF) mRNA has been shown to increase in stromal and epithelial cells of the endometrium in baboons supplemented with estrogen (Niklaus *et al*., 2003). In support of this idea, it has been reported that there is an increase in network formation when uterine microvascular cells are co-cultured with epithelial cells treated with E2 (Albrecht *et al*., 2003).

Targeted gene profiling of angiogenesis factors in each uterine cell line was consistent with observations made in functional assays. In HEECs, pro-angiogenic growth factors were down-regulated, a finding which correlated with the decrease in network formation. In UtMVECs, there was a general up-regulation in pro-angiogenic factors and down-regulation in inhibitors, corresponding with the identified increase in network formation.

Surprisingly, we found that DPN caused a decrease in angiogenesis in HEECs while E2 had no effect. DPN consistently had a strikingly more potent effect on HEEC and UtMVEC network formation than the naturally occurring ligand E2. Additionally, gene expression analysis revealed that treatment of cells with DPN often had a different impact to E2. The planar ligand DPN is both ERβ affinity and potency selective (Meyers *et al*., 2001). The specific chemical structure of DPN means that it docks in the LBD of ERs differently from E2 (Sun *et al*., 2003). Because of these structural differences, these two agonists induce ligand-specific changes in receptor conformation (Leitman *et al*., 2010), precipitating recruitment of different co-regulatory molecules. The nature of the estrogenic ligand is, therefore, the driving force of the composition of co-regulatory complexes, and ultimate ligand-specific gene expression and biological response ensues (Nilsson *et al*., 2011). Our results appear to mirror those in a previous study that compared changes in response to activation of ERβ by the natural product liquiritigenin and those induced by E2 and found only a few common candidates, prompting the authors to state that different ERβ agonists may produce distinct biological effects (Paruthiyil *et al*., 2009). Their study in combination with ours, reporting specific functional effects of DPN on uterine angiogenesis may inform the future use of selective estrogen receptor modulators. If an ERβ-selective agonist can be used to treat endometrial disorders, this would be preferential to the current therapies using GnRH analogues as it would avoid some of the side-effects of an induced hypoestrogenic state.

EC models possess unique ERβ profiles

Targeted gene profiling revealed striking differences in the response of the three EC lines analyzed with very few shared candidates observed. Regulation of three common genes was detected: *HEY1*, *ICAM1*, and *CASP1*. While the two uterine EC lines exhibited very different expression profiles, the HUVEC profile was comparatively unique from either of the other ECs. This reiterates that although HUVECs are used as a common model of vascular function, they do not represent an appropriate substitute in the context of the uterus. Additional genes regulated in the two uterine cell lines were *IFNB1* and *ENPP2*. *ENPP2* has previously been identified as a primary ERβ target gene using chromatin immunoprecipitation in HEK293 (embryonic kidney) cells engineered to overexpress ERβ (Zhao *et al*., 2009). In studies profiling ERβ-dependent genes in malignant cell lines, *ENPP2* was one of the few common genes identified (Monroe *et al*., 2003). In addition to *ICAM1*, *ITGB2* (integrin beta 2) was also regulated in HEECs and UtMVECs, consistent with a role for estrogens in the regulation of vascular permeability (Cho *et al*., 1998). A number of protein families had members that were regulated in two or all of the EC lines. We found that the expression of genes associated with prostaglandin metabolism was altered in all three ECs. In HEECs, thromboxane A synthase 1 (*TBXAS1*) and prostaglandin I2 receptor (*PTGIR*) were reduced, in UtMVECs thromboxane A2 receptor (*TBXA2R*) was increased and, in HUVECs, the cyclooxygenase enzymes (*PTGS1* and *PTGS2*) and *TBXAS1* were all reduced. Estrogen regulates prostanoid synthesis in the placental vasculature (Su *et al*., 2011) and up-regulates the expression of *PTGS1* via ERβ in a mixed population of uterine microvascular cells (Tamura *et al*., 2004). Our results indicate that prostanoid biosynthesis is ERβ regulated in all three ECs, albeit at different steps of the pathway, and possibly with differing outcomes.

ERβ mediates changes in transcription in endometrial ECs via non-classical ER signaling involving the Sp1 transcription factor

In an extension of our observations on gene expression, we also used reporter assays to demonstrate that ERβ was unable to induce transcription via binding to classical EREs in uterine ECs. This finding was consistent with the results obtained by another group performing

studies in HUVECs: they concluded that these cells lacked estrogen responsiveness (Jensen *et al*., 1998), while our studies have shown this is not the case. Bioinformatic analysis revealed that many of our identified ERβ-dependent gene candidates in HEECs had known associations with the transcription factor Sp1. Using immunoprecipitation, we found that ERβ and Sp1 co-exist in a complex in HEECs. We found that the association was not dependent on ligand (DPN) binding to ERβ indicating that the protein complex is pre-formed within the nucleus. Consistent with this hypothesis, our unpublished studies and another report (Muyan *et al*., 2012) have shown that in the absence of ligand, the ERβ protein appears less mobile within the nucleus than ERα suggesting that ERβ is already bound to chromatin regardless of the presence of ligand. As an extension of these findings, we determined whether Sp1 was essential or dispensable for ERβ-dependent changes in gene expression and EC function by using both Mithramycin A, an inhibitor of Sp binding to GC-rich promoter sequences and Sp1-specific siRNA knockdown. Sp1 and ERβ were both found to be essential for DPN-dependent changes in angiogenesis (network formation) and we therefore suggest that DPN induces a conformation in ERβ that favours changes in gene expression via a 'tethered' Sp1-dependent mechanism (Nilsson *et al*., 2001) in endometrial ECs.

Summary

Taken together, gene expression analysis of HEECs, UtMVECs and HUVECs in response to DPN demonstrated: (i) very few common genes but a defined set of unique regulated genes in each cell line; (ii) common genes in HEECs and UtMVECs being regulated in opposing directions; (iii) a general down-regulation of pro-angiogenic factors in HEECs, but a general down-regulation of angiogenesis inhibitors in UtMVECs and (iv) that the latter correlates with the results of functional angiogenesis assays. Moreover, ERβ-mediated changes in gene expression in HUVECs were not similar to that in HEECs or UtMVECs, indicating that this EC type is not suitable for modeling the impacts of ligand-binding ERβ in uterine vascular function. Finally, we showed (v) that the transcription factor Sp1 is required in HEECs for ERβ-dependent changes in gene expression associated with angiogenesis to take place.

Uterine EC models represent paradigms in anatomically diverse microvascular function

We propose that uterine ECs represent good models in which to investigate the potential impacts of ligands capable of high-affinity binding to ERβ in health and disease. Notably, these cells do not express ERα *in vivo* or *in vitro* and so estrogen action must be mediated via ERβ alone. Endometrial ECs were obtained from a tissue subject to cyclical regeneration and the formation of new blood vessels, a quality rare in most adult tissues, the exception being those subject to malignant transformation. We have demonstrated that the ERβ activation in HEECs induces a decrease in network formation by these cells consistent with a reduction in the rate of angiogenesis. In contrast, although also exposed to fluctuating concentrations of estrogens, the vasculature of the normal myometrium is relatively inert although it may be disturbed by the presence of uterine fibroids. We observed an increase in angiogenesis in myometrial ECs in response to ERβ activation, which might complement previous findings that ERβ signaling may have positive effects on angiogenesis within quiescent tissues. Our results suggest that ERβ agonists, both natural, e.g. genestein, and synthetic, e.g. 8b-VE2 and ERb-041, may have unique cell-specific impacts in cells expressing ERβ alone. Although further functional studies are required to validate the therapeutic potential of the above findings, the results presented here may have implications for the *in vivo* application of ERβ subtype-selective ligands.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

E.G. designed and carried out experimental work and wrote the manuscript; F.C. carried out experimental work; H.O.D.C. designed the work; P.T.K.S. designed the work and wrote the manuscript.

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Conflict of interest

The authors have nothing to disclose.

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